Myocardin is sufficient and necessary for cardiac gene expression in *Xenopus*

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Summary

Myocardin is a cardiac- and smooth muscle-specific co-factor for the ubiquitous transcription factor serum response factor (SRF). Using gain-of-function approaches in the *Xenopus* embryo, we show that myocardin is sufficient to activate transcription of a wide range of cardiac and smooth muscle differentiation markers in non-muscle cell types. We also demonstrate that, for the myosin light chain 2 gene (*MLC2*), myocardin cooperates with the zinc-finger transcription factor Gata4 to activate expression. Inhibition of myocardin activity in *Xenopus* embryos using morpholino knockdown methods results in inhibition of cardiac development and the absence of expression of cardiac differentiation markers and severe disruption of cardiac morphological processes. We conclude that myocardin is an essential component of the regulatory pathway for myocardial differentiation.

Key words: Tbx5, Gata4, Nkx2-5, Smooth muscle, Transgenesis

Introduction

Knowledge of the regulatory mechanisms underlying cardiac development is essential for understanding the molecular basis of congenital heart defects. During embryonic heart development, differentiation of cardiac muscle is regulated by interactions between a surprisingly large number of cardiac-restricted transcription factors. For example, the homeodomain transcription factor Nkx2-5 binds directly to the promoters of several cardiac differentiation genes (Chen and Schwartz, 1995; Durocher et al., 1997; Molkentin et al., 2000). However, Nkx2-5 is a relatively weak transcriptional activator and efficient transcription from its target promoters is dependent on the presence of additional regulatory factors. Proteins that interact with Nkx2-5 include the zinc finger transcription factor Gata4 (Durocher and Nemer, 1998; Sepulveda et al., 1998) the T box-containing proteins, Tbx5 and Tbx20 (Bruneau et al., 2001; Stennard et al., 2003) and the homeodomain protein Pitx2 (Ganga et al., 2003). Additional transcription factors, many of which are not cardiac specific, are also essential for embryonic heart development. These include the nuclear factor of activated T cell (NFAT) family of factors (Schubert et al., 2003; Bushdid et al., 2003) and the myocyte enhancer factor 2 (MEF2) family of transcription factors (Molkentin and Markham, 1993; Kuisk et al., 1996; Lin et al., 1997). Each of these proteins are believed to interact with other cardiac transcription factors to control cardiac gene expression (Wada et al., 2002; Black and Olson, 2003).

Numerous cardiac genes also contain binding sites for the ubiquitous transcription factor, serum response factor (SRF). The SRF binding site (the CArG box) has been demonstrated to be essential for myocardial expression of a number of genes including cardiac α-actin (Belaguli et al., 2000; Latinkic et al., 2002), atrial natriuretic factor (Argentin et al., 1994; Small and Krieg, 2003) and the sodium calcium exchanger (Cheng et al., 1999). The presence of CArG elements is not unique to cardiac promoters, however, as binding sites are also common in skeletal and smooth muscle gene promoters, as well as in the control regions of growth factor-inducible genes. Recent studies have shown that SRF activates transcription of smooth and cardiac muscle promoters in collaboration with myocardin, a cofactor that associates directly with SRF but does not bind DNA (Wang et al., 2001). Since myocardin is expressed in cardiac and smooth muscle, but not in skeletal muscle, interactions between myocardin and SRF may provide the mechanism by which cardiac and smooth muscle-specific promoters are distinguished from skeletal muscle promoters. Recent studies have emphasized the role of myocardin as a powerful activator of smooth muscle genes (Chen et al., 2002; Du et al., 2003; Wang et al., 2003) and the mouse knockout of myocardin results in embryonic death due to absence of vascular smooth muscle differentiation (Li et al., 2003). In

many respects therefore, myocardin has the properties of a master regulator of smooth muscle development.

The lack of a cardiac phenotype in myocardin knockout mice is seemingly at odds with our previous studies which showed that expression of a dominant negative of myocardin in *Xenopus* embryos was able to abolish cardiac gene expression, suggesting an important role for myocardin in heart development (Wang et al., 2001). However, a caveat in the interpretation of such dominant negative experiments is that such mutants can interfere with multiple transcriptional regulators. It has been proposed that redundant activities of the myocardin-related factors, MRTF-A and MRTF-B (Wang et al., 2002) may be sufficient to rescue heart development in myocardin mutant embryos but this possibility has not yet been addressed experimentally.

In this study, we show that myocardin is able to activate a large number of cardiac and smooth muscle differentiation genes in non-muscle cells. We also show that myocardin can function combinatorially with another cardiac-expressed transcription factor, Gata4, to achieve efficient transcription of cardiac differentiation markers. Conversely, depletion of myocardin in the developing embryo by antisense morpholino injection abolishes cardiac marker gene expression, indicating that myocardin is essential for regulation of cardiac differentiation.

Materials and methods

Embryological manipulations

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). *Xenopus* eggs were in vitro fertilized, dejellied using 2% L-cysteine (pH 8.0), and maintained in 0.2× MMR (Marc's Modified Ringers). Targeted mRNA injections were performed in 0.4× MMR/4% Ficoll at the 8-cell stage. Different mRNAs (125 pg, 250 pg or 400 pg) were injected in a volume of 4.6 nl. Embryos were cultured in 0.2× MMR until a suitable stage and fixed in MEMPFA for assay by in situ hybridization. In animal cap assays embryos were injected at the one-cell stage into the animal pole. Animal pole explants were dissected from mRNA-injected or uninjected control embryos at stage 8 in 1× NAM (Normal Amphibian Medium). Caps were cultured in 0.5× NAM containing gentamycin until the equivalent of stage 12.5 and RNA was extracted for RT-PCR analysis.

Xenopus laevis transgenesis

The *NβT* promoter driven myocardin or GFP transgene was linearized with *Pme*I and transgenic *Xenopus* embryos were generated using previously described methods (Kroll and Amaya, 1996; Sparrow et al., 2000; Small and Krieg, 2003). Double transgenics were made using the *NβT-GFP* and *NβT-myocardin* constructs, and GFP expression in neural tissues was used as a control for transgenesis.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out using a modification of the protocol of Harland (Harland, 1991), using antisense digoxigenin-labeled probes transcribed using a MEGAscript kit (Ambion). For serial sections, embryos were post-fixed in 4% paraformaldehyde for 6 hours at room temperature and embedded in Paraplast. Transverse sections (10 μm) were cut on a microtome.

Cloning of *Xenopus laevis myocardin*

RT-PCR using degenerate primers designed against the conserved amino acid regions WETMEWL and IFNIDF within the human and mouse myocardin sequences was performed using random-primed adult *Xenopus* heart cDNA. After cloning and sequencing of the resulting 99 bp fragment, the remaining portions of the myocardin cDNA were amplified by 5′ and 3′ RACE using *Xenopus* heart cDNA prepared according to the manufacturer's instructions (FirstChoice RLM-RACE Kit; Ambion). The final *Xenopus myocardin* sequence was determined by PCR amplification, cloning and sequencing of the entire myocardin coding region using the Expand High Fidelity PCR System (Roche).

RT-PCR of marker gene expression

Ten animal cap explants were harvested for each sample and RNA was isolated using buffer A/proteinase K. cDNA was prepared from one half of each RNA sample, and a minus RT negative control sample was prepared from the remaining RNA. One fiftieth of the cDNA sample was used as template in radioactive RT-PCR that included 0.3 μCi of α-^{32}P in a 20 μl reaction. RT-PCR cycle number was determined to assure the reaction was in the linear range of amplification. PCR samples were separated on non-denaturing 5% acrylamide gels.

Primers

Primers used were as follows: calponin H1, 5′-GCACTGTACGGAAGATCAACG-3′ (forward) and 5′-CGATATCCACTCTGGCACCTT-3′ (reverse) (Tm=60°C); cardiac α actin (Niehrs et al., 1994) (Tm=63°C); cTnI (Vokes and Krieg, 2002) (Tm=63°C); Gata4, 5′-TCTGGCCACAACATGTGG-3′ (forward) and 5′-CAGTTGACACATTCTCGG-3′ (reverse) (Tm=56°C); Mef2A, 5′-CAGCTCCAGCAGTTCCTAT-3′ (forward) and 5′-TTACACTGAGGCCTAATGCA3′ (reverse) (Tm=56°C); MHCα, 5′-ACCAAGTACGAGACTGACGC-3′ (forward) and 5′-CTCTGACTTCAGCTGGTTGA-3′ (reverse) (Tm=60°C); MLC2, 5′-GAGGCATTCAGCTGTATCGA-3′ (forward) and 5′-GGACTCCAGAACATGTCATT-3′ (reverse) (Tm=60°C); MRF4, 5′-ATCAGCAGGACAAGCCACAGA-3′ (forward) and 5′-TGTATAGTGCAAGGGTGCCTG-3′ (reverse) (Tm=58°C); MRTF-A, 5′-TGAGGGTAAGCAAAAATTAAG-3′ (forward) and 5′-GGTGAACTGAAGTGCCATAGA-3′ (reverse) (Tm=60°C); MRTF-B, 5′-TTGGGTGCATAGCTGGAACT-3′ (forward) and 5′-CCAAGCATCATCCTGTGTTAC-3′ (reverse) (Tm=60°C); Myf5, 5′-CATGTCTAGCTGTTCAGATGG-3′ (forward) and 5′-CAATCATGCGCATCAGGTGAC-3′ (reverse) (Tm=58°C); MyoD, 5′-AACTGCTCCGATGGCATGATGGATTA-3′ (forward) and 5′-ATTGCTGGGAGAAGGGATGGTGATTA-3′ (reverse) (Tm=60°C); myocardin, 5′-GCCCAAAGCAAATTACAAGAA-3′ (forward) and 5′-GGAAGTCGGTGTTGAAGATAC-3′(reverse) (Tm=60°C); myogenin, 5′-CCTGAATGGAATGACTCTGAC-3′ (forward) and 5′-GGCAGAAGGCATTATATGGAA-3′ (reverse) (Tm=58°C); Nkx2-5, 5′-TCTGAACTCACTGAGGAA-3′ (forward) and 5′-AGGACTGGTACAGCTATC-3′ (reverse) (Tm=56°C); ODC (Bouwmeester et al., 1996) (Tm=64°C); SkMLC, 5′-TGGTCAAAGAGGCATCTGGA-3′ (forward) and 5′-AGTTGTGTCTCTGATGGGATG-3′ (reverse) (Tm=60°C); SM22, 5′-TCCAGACAGTAGACCTGTATG-3′ (forward) and 5′-GTCGACCGTATCCTGTCATC-3′ (reverse) (Tm=60°C); SM actin, 5′-ACCACTTACAACAGCATCATG-3′ (forward) and 5′-ACCAATCCAGACGGAGTACTT-3′ (reverse) (Tm=60°C); SRF, 5′-TGCACTGTGCCTGTGTGATTA-3′ (forward) and 5′-CAGACTCACACAACTTGCACA-3′ (reverse) (Tm=58°C); Xbra (Vokes and Krieg, 2002) (Tm=60°C).

Myocardin loss of function by morpholino oligonucleotide injection

Antisense morpholinos (MO1 5′-CAGCTTTTCTGGTTTAATGGTTTAT-3′ and MO2 5′-TGTTCGGAACCCAAGAGAGTCATGT-3′) were directed against two independent sequences near the 5′ end of the *myocardin* transcript. The morpholinos were targeted to sequences that are identical in the A and B copies of the *Xenopus laevis* genes in order to inhibit translation of both mRNAs. A dose curve was determined with 2.5 ng, 5 ng, 10 ng and 20 ng of

morpholino, injected in one cell of a two-cell embryo so the uninjected side served as a negative control. A concentration-dependent phenotype was observed with an increasing percentage of asymmetric cardiac gene expression with increasing dose. MO-treated embryos were assayed using in situ hybridization and appropriate marker probes.

Results

Cloning and expression of *Xenopus myocardin*

The deduced amino acid sequence of the *Xenopus laevis* myocardin protein is presented in Fig. 1, aligned with the mouse and human myocardin protein sequences. *Xenopus* myocardin contains 918 amino acids with a predicted molecular mass of 101 kDa. We conclude that this protein represents the *Xenopus* orthologue of myocardin for the following reasons. First, *Xenopus* myocardin is 56% and 57% identical to the mouse and human proteins, respectively, and this level of sequence identity is similar to that of other regulatory proteins in the cardiogenic pathway, such as Gata4 and Nkx2-5. Second, the sequence identity between *Xenopus* myocardin and mouse myocardin-related factors, MRTF-A and B (MKL1 and MKL2 – Mouse Genome Informatics), is only 32% and 31% respectively. Third, a search of the draft *Xenopus* genomic sequence revealed no other genes with greater similarity to mammalian myocardin. Finally, the basic region of myocardin, which is involved in interactions with SRF (Wang et al., 2001) and the SAP domain, which is thought to function in chromatin remodeling (reviewed by Aravind and Koonin, 2000) are both highly conserved between frog and mouse (89% and 90%, respectively).

The expression pattern of *myocardin* during *Xenopus*

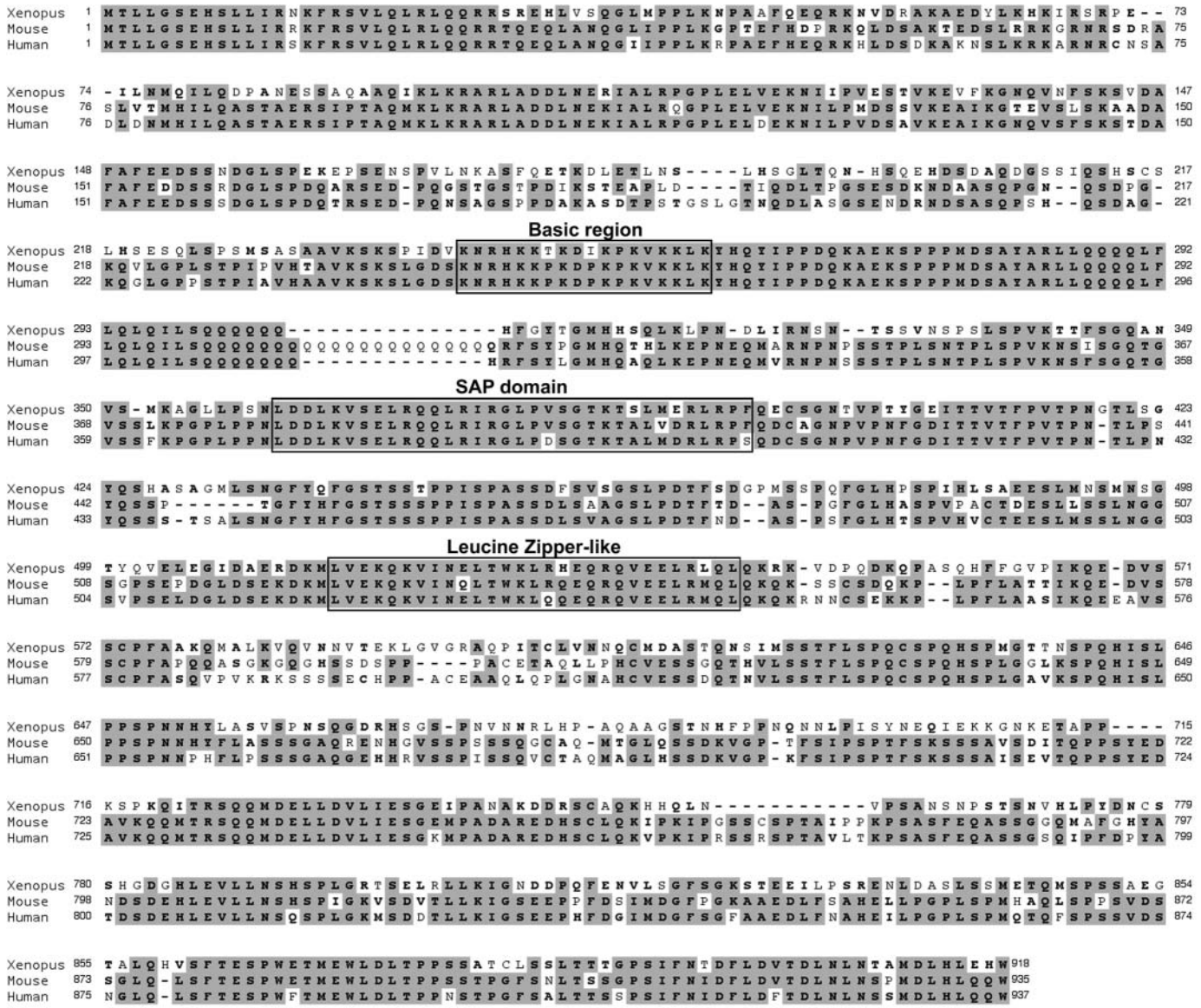

Fig. 1. Alignment of frog, mouse and human myocardin proteins. The basic region (involved in SRF binding), the SAP domain (involved in chromatin remodeling), and the leucine zipper-like domain (dimerization domain), are labeled. The *Xenopus* myocardin protein is 56% and 57% identical to the mouse and human proteins, respectively. Accession number for *Xenopus laevis* myocardin is AY585230.

development has been determined using whole-mount in situ hybridization (Fig. 2). *Myocardin* transcripts are not detectable in the early neurula embryo (stage 15; Fig. 2A) at a time when transcripts for the precardiac marker, *Nkx2-5*, are abundant (Fig. 2C). *Myocardin* expression is first detected in the late neurula embryo (stage 24) in the pre-cardiac patches of anterior lateral mesoderm (Fig. 2A'). This expression precedes, by approximately 3 hours, detection of transcripts for *myosin heavy chain-α* (*MHCα*) which is an early and robust marker of myocardial differentiation in the *Xenopus* embryo. In the mouse embryo, *myocardin* is co-expressed with *Nkx2-5* in early cardiac primordia (Wang et al., 2001) but this does not appear to be the case in the frog embryo. At stage 27, *myocardin* transcript levels continue to increase (Fig. 2A'') and *MHCα* transcripts are detectable in the differentiating cardiac tissues (Fig. 2B''). At this stage the domain of *myocardin* expression appears identical to that of *MHCα*, but is significantly more restricted than the *Nkx2-5* expression domain (Fig. 2A''',B''',C'''). *Myocardin* expression persists during subsequent cardiac development and is visible throughout the atrial and ventricular muscle layers of the tadpole heart (Fig. 2D). By the tadpole stage (stage 40), *myocardin* transcripts are also visible in visceral smooth muscle cells surrounding the looping gut (Fig. 2E,F) and in isolated smooth muscle precursor cells adjacent to the forming dorsal aorta (Fig. 2F). Overall, the expression of *Xenopus myocardin* in developing cardiac and smooth muscle tissues closely resembles the expression profile reported for the murine *myocardin* gene (Wang et al., 2001). Two additional *Xenopus* myocardin-related sequences, *MRTF-A* and *MRTF-B* have been reported (Wang et al., 2002) and in situ hybridization analysis of these sequences is shown in Fig. 2G and H, respectively. Neither gene shows detectable expression in the cardiogenic region of the embryo, even when the chromogenic detection reaction is continued until non-specific background staining becomes evident. RT-PCR analysis indicates that, surprisingly, *myocardin*, *MRTF-A* and *MRTF-B* transcripts are all present at significant levels in the fertilized egg, but transcripts decline to effectively undetectable levels by the gastrula stage. RT-PCR analysis of isolated heart patch tissue from the stage 28 embryo shows abundant expression of *myocardin* but no detectable expression of *MRTF-A* or *B* (Fig. 2I). The absence of *MRTF-A* and *B* transcripts from the pre-cardiac region is important for interpretation of morpholino knockdown experiments described below.

Myocardin induces ectopic cardiac muscle gene expression in whole embryos

Previous studies using cells in culture showed that myocardin is able to activate reporter genes containing a range of myocardial (Wang et al., 2001; Wang et al., 2002) and smooth muscle promoters (Chen et al., 2002; Wang et al., 2003; Du et al., 2003). Myocardin is also able to activate expression of

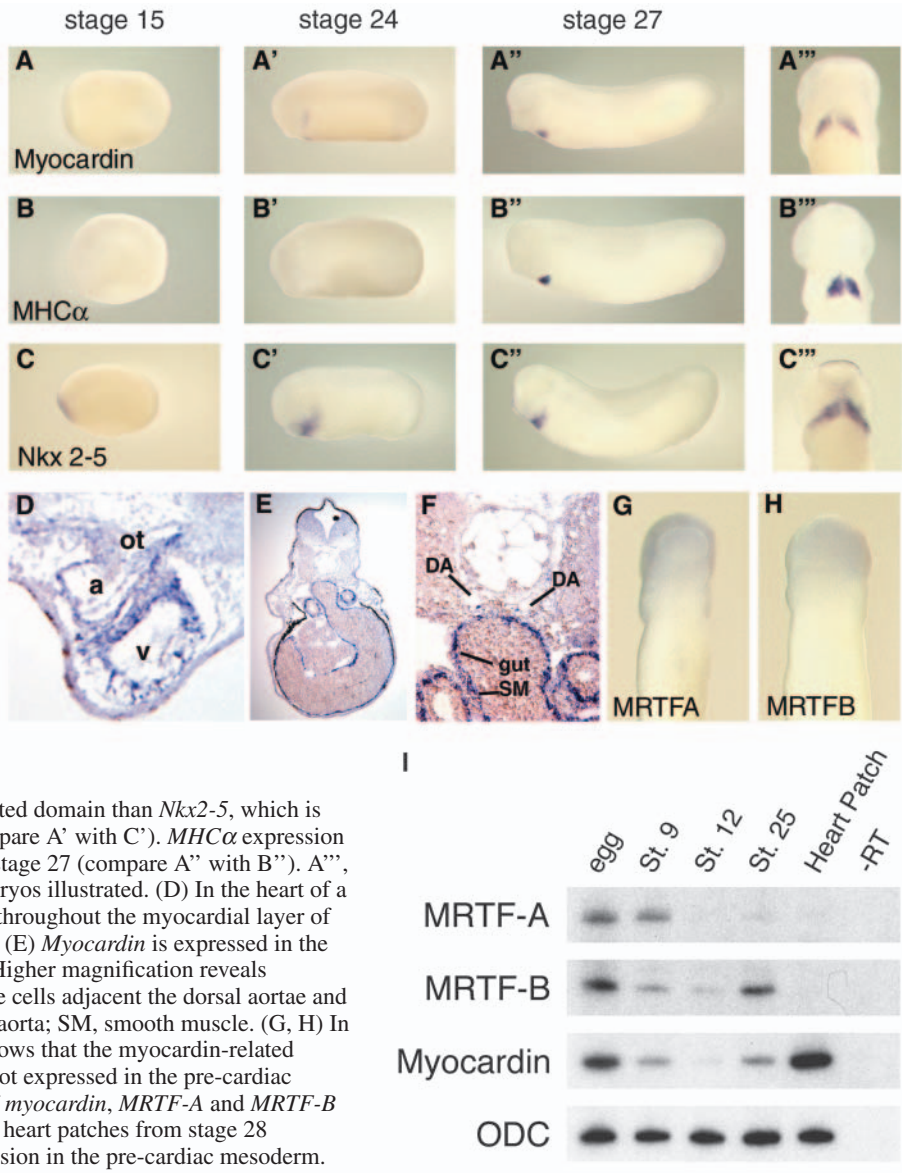

Fig. 2. Developmental expression of *Xenopus myocardin* and *MRTF* genes. The expression of *Xenopus myocardin* (A-A''') was analyzed by whole-mount in situ hybridization and compared to the expression patterns of the cardiac differentiation marker, *MHCα* (B-B'''), and the pre-cardiac marker, *Nkx2-5* (C-C''') at the stages indicated. *Myocardin* expression in the stage 24 embryo is localized to the pre-differentiation cardiac mesoderm in a more restricted domain than *Nkx2-5*, which is also expressed in the pharyngeal arch region (compare A' with C'). *MHCα* expression is located in an identical domain to myocardin at stage 27 (compare A'' with B''). A''', B''' and C''' are ventral views of the stage 27 embryos illustrated. (D) In the heart of a stage 45 embryo *myocardin* expression is located throughout the myocardial layer of the atrium (a), ventricle (v), and outflow tract (ot). (E) *Myocardin* is expressed in the visceral smooth muscle in stage 42 embryos. (F) Higher magnification reveals *myocardin* expression in individual smooth muscle cells adjacent the dorsal aortae and in the smooth muscle layer of the gut. DA, dorsal aorta; SM, smooth muscle. (G, H) In situ hybridization analysis of stage 27 embryos shows that the myocardin-related transcription factors, *MRTF-A* and *MRTF-B*, are not expressed in the pre-cardiac mesoderm (ventral views). (I) RT-PCR analysis of *myocardin*, *MRTF-A* and *MRTF-B* expression in early *Xenopus* embryos and isolated heart patches from stage 28 embryos confirms a lack of *MRTF-A* and *B* expression in the pre-cardiac mesoderm.

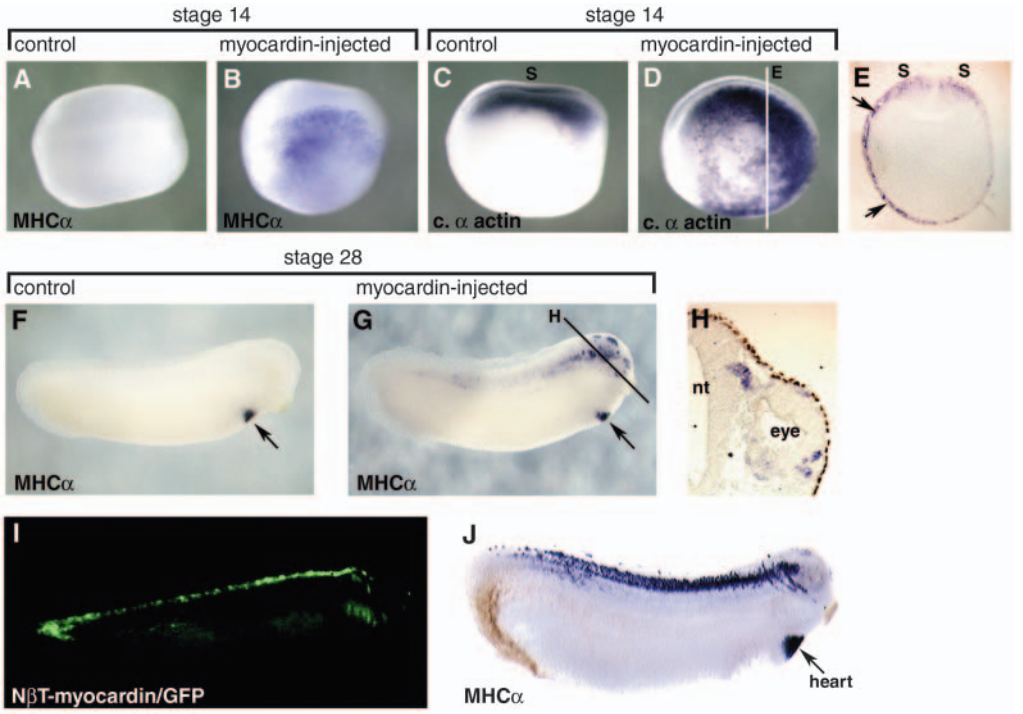

Fig. 3. Myocardin activates ectopic expression of myocardial markers in the *Xenopus* embryo. (A-H) 125 pg of *myocardin* mRNA was injected into one cell of an eight-cell embryo, which was then assayed for cardiac markers by whole-mount in situ hybridization. No expression of the *MHCα* gene is observed in uninjected stage 14 embryos (A), however widespread transcription of *MHCα* is observed in *myocardin*-injected embryos (B). Similarly, *cardiac α-actin* is observed specifically in the pre-somitic mesoderm at stage 14 control embryos (C), while myocardin injected embryos display widespread expression of *cardiac α-actin* on the side of injection (D). (E) Section through the embryo in D shows ectopic *cardiac α-actin* expression (arrows) in the ectodermal and mesodermal tissue layers. Ectopic cardiac marker expression is not observed in endodermal tissues. (F) *MHCα* expression is heart-specific at stage 28 in un-injected control embryos, but myocardin overexpression, (G), causes *MHCα* transcription in ectopic locations. Arrows indicate normal cardiac expression. (H) Section through the embryo in G shows patches of ectopic *MHCα* expression in the neural tube (nt) and eye. (I) Fluorescence microscopy of a stage 29 *Xenopus* embryo co-transgenic for *NβT*-GFP and *NβT*-myocardin showing GFP expression in neural tissues. (J) In situ hybridization analysis of *NβT*-GFP/*NβT*-myocardin co-transgenic embryos using a *MHCα* probe shows ectopic expression of *MHCα* in neural tissues.

endogenous smooth muscle genes (Chen et al., 2002; Du et al., 2003; Wang et al., 2003; Yoshida et al., 2003). We used the *Xenopus* embryo as an in vivo model system to investigate the potential of myocardin to regulate transcription of endogenous cardiac and smooth muscle genes. In these experiments, mRNA encoding myocardin was injected into single blastomeres of eight-cell embryos. The uninjected side of the embryo served as a negative control. At subsequent stages of development, expression of cardiac or smooth muscle markers was determined by in situ hybridization. Our results demonstrate that myocardin is sufficient to induce precocious and ectopic expression of cardiac markers. For example, expression of the cardiac-specific differentiation marker, *MHCα*, is normally initiated in the late neurula embryo (stage 25) and is undetectable at stage 14 (Fig. 3A). In embryos injected with *myocardin* mRNA however, high levels of ectopic *MHCα* transcripts were present in stage 14 embryos (Fig. 3B) approximately 24 hours before expression would normally be detected in the heart. Ectopic expression was observed in 65% of myocardin-injected embryos (13/20). Similarly, precocious and ectopic expression of *cardiac α-actin* was observed in myocardin-injected embryos (Fig. 3D,E) as was somewhat weaker expression of *cardiac troponin I* (*cTnI*), and the smooth muscle marker *SM22* (data not shown). Ectopic expression of *MHCα* persisted through subsequent development, and appeared to be particularly strong in neural tissues (Fig. 3G). As shown in Fig. 3H, *MHCα* transcripts could be detected in isolated patches, apparently within tissues of the eye and neural tube. Examination of numerous sectioned embryos suggested that ectopic expression of cardiac markers was limited to ectodermal and mesodermal tissue layers because we never observed cardiac gene transcripts in endoderm derivatives, even when myocardin mRNA was specifically targeted to this germ layer. Finally, despite extended culturing of myocardin mRNA injected embryos, we never observed the presence of beating tissue or striated muscle at ectopic locations in the embryo.

While these embryo injection results show that myocardin is capable of activating ectopic cardiac marker expression in the embryo, it is important to note that not all cardiac differentiation markers were induced. For example, we never observed ectopic expression of *myosin light chain-2* (*MLC2*) transcripts in these embryos, which is surprising since the *MLC2* gene is regulated by SRF (Qasba et al., 1992; Latinkic et al., 2004) and because myocardin activates the *MLC2* promoter in cultured cells (Wang et al., 2001). The failure of myocardin to activate *MLC2* in *Xenopus* does not seem to be a dose effect, since injection of greater amounts of myocardin mRNA did not succeed in activating *MLC2* expression.

Transgenic expression of myocardin activates cardiac gene expression in neural tissues

When mRNA is injected into a *Xenopus* embryo, translation of the mRNA commences almost immediately. In the experiments described above, the embryos were injected at the eight-cell stage, however, activation of the first tissue-specific transcription pathways did not commence until approximately the gastrulation stage of development (stage 10). It is possible

therefore, that myocardin was only capable of initiating cardiac gene expression ectopically, in the absence of competing developmental programs. To address this issue, we generated transgenic embryos in which transcription of myocardin mRNA was driven by the *neural β tubulin* (*NβT*) promoter. *NβT* is a neural differentiation marker that is specifically expressed in the central and peripheral nervous system (Richter et al., 1988) and an *NβT*-GFP transgene recapitulates the endogenous expression pattern (Kroll and Amaya, 1996) (Fig. 3I). A neural promoter was chosen for these experiments because *Xenopus* neural tissues express high levels of the essential myocardin cofactor, SRF (data not shown) and because we sought to determine whether myocardin was capable of activating cardiac gene expression in cells derived from the ectodermal germ layer. Embryos expressing myocardin in neural tissues were assayed by in situ hybridization for *MHCα* transcripts. As shown in Fig. 3J, *MHCα* expression was activated throughout differentiated neural tissues, in a pattern identical to that of the GFP marker (Fig. 3I). Moreover, the level of *MHCα* expression in the neural tube was comparable to the level of expression of the endogenous gene in the heart. This result indicates that myocardin is able to activate transcription of cardiac-specific genes in tissues that are already specified to a neural fate. Transgenic embryos expressing myocardin in neural tissues developed normally and showed a full range of reflex responses, suggesting that myocardin did not subvert normal neural development.

Myocardin activates cardiac and smooth muscle differentiation markers in animal cap explants

Since myocardin is able to activate cardiac tissue markers in whole *Xenopus* embryos, we wished to assess its ability to activate myocardial gene transcription in a more defined system. Animal cap explants from the *Xenopus* embryo, consisting entirely of naive ectodermal tissue, have been widely used to investigate gene expression (Cascio and Gurdon, 1987; Grainger and Gurdon, 1989; Howell and Hill, 1997; Tada et al., 1998) and are a convenient alternative to cultured cells. Animal caps typically differentiate to form epidermal tissue and never express cardiac genes (Fig. 4A, lane labeled uninjected). Animal cap tissue contains a significant amount of *SRF* mRNA (Fig. 4A) and so the essential myocardin cofactor is present in these cells. The consequences of expressing myocardin in animal cap explants was assayed at stage 12.5, corresponding to the late gastrula stage and approximately 24 hours before myocardial marker expression would commence in the intact embryo. As shown in Fig. 4A, myocardin precociously activates a range of myocardial differentiation markers including *MHCα, cTnI* and *cardiac α-actin* (which is also expressed in skeletal muscle). In addition, myocardin activated expression of the smooth muscle differentiation markers *SM actin*, *calponin H1* and *SM22* (Fig. 4A).

As in the whole embryo experiments, myocardin did not activate expression of all myocardial genes. Of the markers tested, *MLC2* was never expressed in animal caps (Fig. 4A), even when the dose of myocardin was increased approximately fourfold over the amount sufficient to activate *MHCα* (data not shown). SRF was not limiting in these experiments since co-injection of SRF mRNA, together with myocardin mRNA, did not alter the results (data not shown). It has been reported that expression of Gata4 in animal cap explants is sufficient to initiate the complete cardiac differentiation pathway, including formation of beating tissue (Latinkic et al., 2003). In that study, cardiac marker expression was first observed at about stage 28, corresponding to the normal time at which cardiac markers are observed in the intact embryo. To test whether more time might be required for expression of *MLC2* we also examined animal cap explants cultured until stage 29-30. In all cases, the expression of markers at stage 29 was identical to that observed at stage 12.5, indicating that time of culture is not a significant factor in these experiments (data not shown).

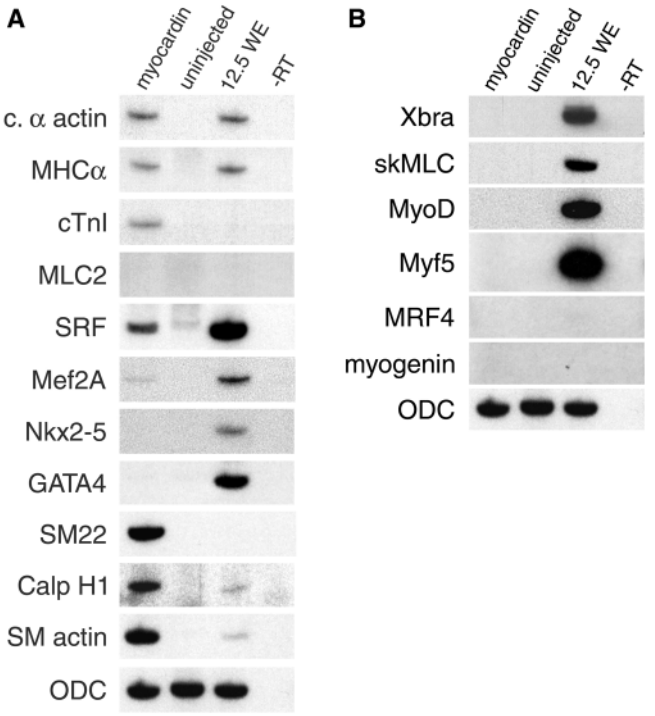

Fig. 4. Myocardin induces transcription of endogenous cardiac and smooth muscle marker genes in animal cap explants. Myocardin-expressing animal pole explants were cultured until stage 12.5 and assayed for cardiac and smooth muscle gene expression by RT-PCR. (A) Uninjected animal caps differentiate into epidermal tissue and never express mesodermal derivatives, including cardiac or smooth muscle markers (lane labeled uninjected). Myocardin-injected caps however, express a wide range of cardiac and smooth muscle differentiation markers (lane labeled myocardin), including *cardiac α-actin*, *MHCα, cardiac TnI*, *SM22*, *calponin H1* and *smooth muscle actin*. The myocardin cofactor *SRF* and the MADS box transcription factor *Mef2a,* are upregulated in myocardin expressing caps. The cardiogenic genes, *Nkx2-5* and *Gata4* are not expressed in myocardin-injected animal caps. The lane labeled 12.5 WE, represents the normal expression of the assayed genes in the whole embryo at the time that the animal cap explants were assayed. (B) Myocardin does not activate genes of the skeletal muscle or mesodermal pathways. Myocardin-injected animal caps were assayed by RT-PCR for the activation of mesodermal and skeletal muscle markers. The general mesoderm marker *brachyury* (*Xbra*) is not expressed in myocardin-injected caps. Furthermore, myocardin does not activate expression of the skeletal muscle transcription regulators, *MyoD*, *Myf5*, *MRF4* and *myogenin*, or the skeletal muscle-specific differentiation marker *skMLC*.

These results suggest that, although myocardin is able to precociously activate transcription of a subset of myocardial markers, it is not sufficient to initiate the complete cardiac development program. Investigation of the expression of other cardiogenic genes supports this proposal. First, transcription of the *Nkx2-5* or *Gata4* transcription factors was not activated in response to myocardin (Fig. 4A). Both of these genes are essential for normal cardiogenesis (Lyons et al., 1995; Tanaka et al., 1999; Molkentin et al., 1997). We note, however, that expression of SRF and the MADS box transcription factor, *Mef2A*, were both activated in animal caps (Fig. 4A), indicating that at least some cardiac regulatory factors lie downstream of myocardin and may play a role in ectopic activation of cardiac markers. Transcription of the *myocardin* gene itself, however, was not activated in animal caps (data not shown), indicating that myocardin does not directly regulate its own expression. We also addressed the possibility that the marker gene expression observed in response to myocardin might be due to activation of the skeletal muscle pathway. This is particularly relevant for the *cardiac α-actin* marker, which is expressed in both cardiac and skeletal muscle tissues in the embryo. RT-PCR analysis showed that no transcripts were present for the general mesoderm marker, *brachyury (Xbra)*, the myogenic determination genes *MyoD*, *Myf5*, *MRF4* or *myogenin*, nor for the skeletal muscle marker, *skMLC* (Fig. 4B), demonstrating that the skeletal muscle program was not activated in the animal cap explants.

Myocardin cooperates with cardiogenic factors to regulate transcription

The failure to observe *MLC2* transcription in animal caps was unexpected since the MLC2 promoter is regulated by myocardin in transfection assays using COS cells (Wang et al., 2001). One possible explanation for these findings is that activation of *MLC2* expression may require transcription factors, in addition to myocardin, that are not present in animal cap cells. Therefore, we tested the ability of Gata4, Nkx2-5 and Tbx5, all of which are important regulators of cardiac gene expression (Durocher et al., 1997; Chen and Schwartz, 1996; Lyons et al., 1995; Tanaka et al., 1999; Bruneau et al., 2001) to cooperate with myocardin in activation of *MLC2* expression. Mixtures of mRNAs encoding all four factors were tested in the animal cap assay at stage 12.5 (Fig. 5A). First, we observed that co-expression of all four transcription factors succeeded in activating expression of *MLC2* (lane labeled M+N+G+T). This activation was not observed using a mixture of the three transcription factors in the absence of myocardin (lane marked N+G+T). Second, the presence of all four transcription factors did not significantly increase *MHCα* or *SM22* expression levels relative to myocardin alone, suggesting that the other factors are not required for efficient expression of these genes in the animal cap. Third, no beating tissue was observed in animal caps co-expressing all four transcription factors, even when the explants were cultured until the equivalent of stage 45, approximately 5 days after a beating heart would form in the intact embryo (data not shown). This result indicates that the presence of this particular combination of factors is not sufficient to activate the complete pathway leading to myocardial differentiation. Testing of myocardin with different combinations of transcription factors (Fig. 5B), revealed that the expression of myocardin and Gata4 alone was sufficient to activate *MLC2* transcription in the animal cap. This result is consistent with recent transgenic studies of the *Xenopus MLC2* promoter that show essential roles for SRE and GATA regulatory elements (Latinkic et al., 2004).

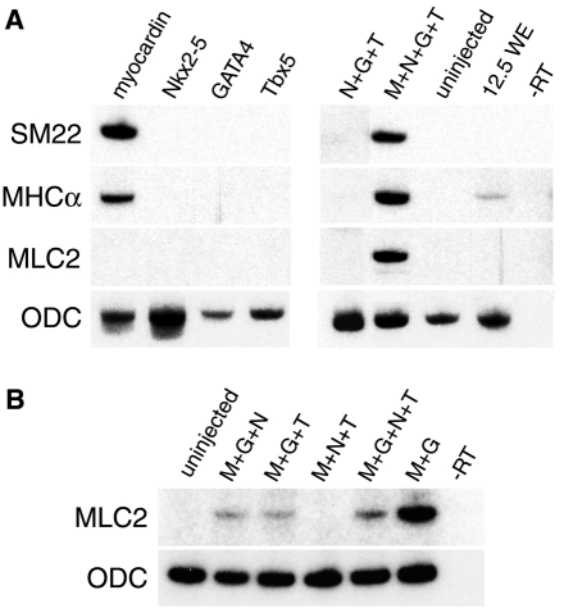

Fig. 5. Myocardin acts in combination with other cardiac transcription factors to activate endogenous *MLC2* expression in animal cap explants. (A) Expression of myocardin alone (lane labeled myocardin) activates SM22 and MHCα expression, but is not sufficient to activate expression of the *MLC2* gene. Expression of Nkx2-5, Gata4, or Tbx5 alone, or the combination of these three factors (lane labeled N+G+T) is not sufficient to activate expression of *MLC2* or *SM22* or *MHCa*. However, when myocardin is coexpressed with Nkx2-5, Gata4 and Tbx5, *MLC2* gene expression is activated (lane labeled M+N+G+T). M, myocardin; N, Nkx2-5; G, Gata4; T, Tbx5. (B) Co-expression of combinations of transcription factors in animal cap explants shows that any combination of myocardin and Gata4 is sufficient to activate *MLC2* expression.

Myocardin loss of function by morpholino knockdown results in a block to cardiac differentiation

To determine whether myocardin is essential for expression of cardiac genes in the developing embryo, we utilized the antisense morpholino method for inhibition of translation of specific mRNAs. It was only possible to examine cardiac marker expression in these experiments because smooth muscle differentiation occurs rather late in *Xenopus* development (after about stage 35) and is therefore outside of the window for morpholino interference (Heasman et al., 2000). Two independent morpholinos, MO1 and MO2, complementary to non-overlapping sequences within the 5′ end of the *Xenopus* myocardin mRNA were prepared and control experiments demonstrated that these effectively inhibited translation of a myocardin fusion transcript in the embryo (Fig. 6A,B). Based on limited sequence conservation, neither of these morpholinos is expected to inhibit translation of *MRTF-A* or *B*. For loss-of-function experiments, MO1 was injected into one cell of a two-cell embryo, so that the uninjected side served as a stage-matched negative control. The injected

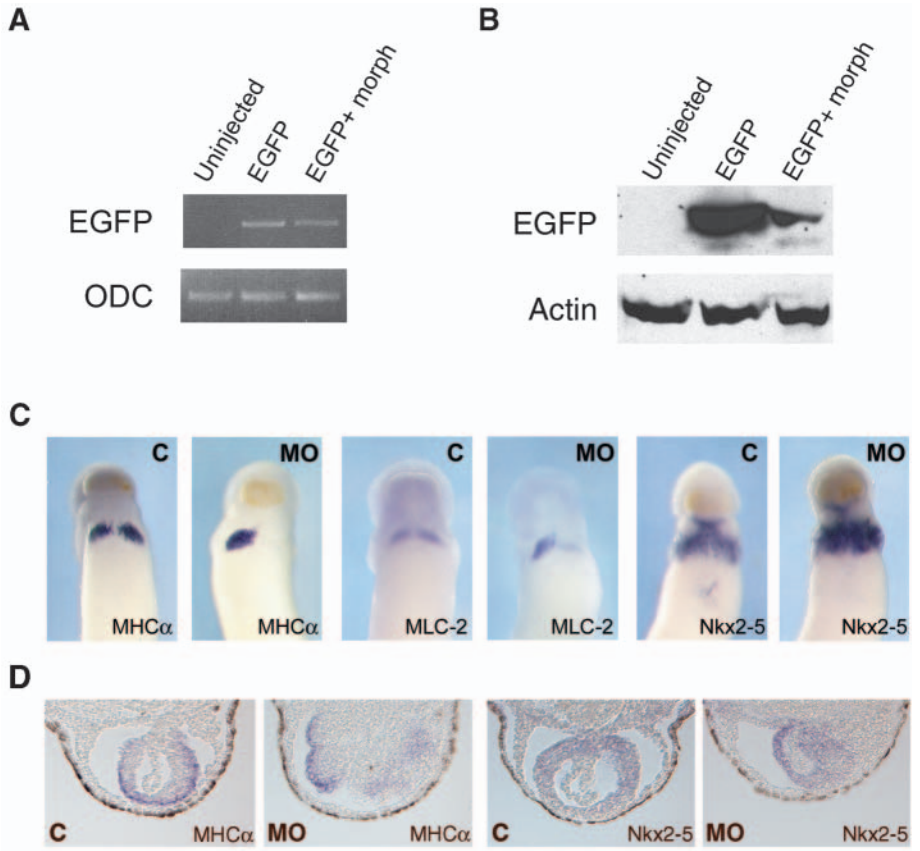

Fig. 6. Inhibition of myocardin activity using antisense morpholino (MO) oligos. (A,B) Control experiment where myocardin MO1 inhibits translation of a transcript containing the myocardin 5′UTR fused to the EGFP coding region. mRNA (400 pg) was injected into one-cell *Xenopus* embryos with or without 10 ng of myocardin MO1 and the embryos were then assayed for the presence of GFP transcript and protein at stage 17. The presence of MO1 did not affect the levels of EGFP transcript as detected by RT-PCR (A) but did significantly reduce the amount of translated GFP protein as detected by western blotting (B). (C) *Xenopus* embryos were injected with 10 ng of myocardin MO1 into one blastomere at the two-cell stage and cultured until stage 29, when cardiac differentiation markers are normally expressed in the symmetric heart patches. Uninjected control embryos (labeled C) or myocardin MO1-injected embryos (labeled MO) were assayed by in situ hybridization. Myocardin MO1 inhibited expression of *MHCα* and *MLC2* on the side of injection (right side of figure) but did not affect the expression of *Nkx2-5*. (D) Sections through the heart of uninjected (labeled C) and one-sided MO1-injected (labeled MO) *Xenopus* embryos at the linear heart tube stage (stage 34). Embryos were assayed by in situ hybridization for expression of either *MHCα* or *Nkx2-5* transcripts to mark the location of myocardial cells and to confirm a reduction in *MHCα* expression on the injected side (right side) of the MO-injected embryo. Uninjected controls showing normal heart tube morphogenesis are included for comparison.

embryos were then raised until stage 28-29 when they were assayed for cardiac marker gene expression by in situ hybridization. Injection of 10 ng of MO1 resulted in a significant inhibition of *MHCα* expression on the side of injection (Fig. 6C and Table 1). Inhibition of *MHCα* expression was observed in 57% of experimental embryos (13/23). Using *MLC2*, an independent marker of cardiac differentiation, inhibition was observed in 86% of embryos (42/49). Asymmetry of cardiac marker expression was observed in only 4-5% of uninjected controls (Table 1). The independent morpholino sequence, MO2, also inhibited expression of *MHCα* and *MLC2*, although the efficiency was somewhat less than that observed for MO1 (Table 1). In situ hybridization detection of *Nkx2-5* transcripts showed that expression of this precardiac marker sequence was unaffected by myocardin MO treatment (Fig. 6C). Similarly, Gata4 levels were not influenced by the presence of the myocardin MO (data not shown). These results indicate that precardiac tissues are still present in MO-treated embryos and that the *Nkx2-5* and *Gata4* expression pathways are independent of myocardin activity. Moreover, transverse sections of control and myocardin MO-treated embryos at the linear heart tube stage (st. 34) revealed that the normal morphogenic movements associated with heart tube formation (i.e. delamination) are disrupted on the MO-injected side while the uninjected side of the embryo appears normal (Fig. 6D). Overall, these loss-of-function studies show that myocardin activity is essential for expression of cardiac differentiation markers and for cardiac morphogenic movements during *Xenopus* development.

Table 1. Summary of myocardin morpholino phenotypes

Morpholino	Normal cardiac gene expression	Reduced/ eliminated expression	Number of embryos
MHCα expression			
Control	142 (95%)	8 (5%)	150
MO1	10 (43%)	13 (57%)	23
MO2	87 (64%)	48 (36%)	135
MLC2 expression			
Control	48 (96%)	2 (4%)	50
MO1	7 (14%)	42 (86%)	49
MO2	29 (60%)	20 (40%)	49
Nkx2-5 expression			
Control	49 (98%)	1 (2%)	50
MO1	50 (91%)	5 (9%)	55
MO2	20 (100%)	0 (0%)	20

Discussion

Myocardin activates endogenous expression of cardiac differentiation markers

Several recent papers have emphasized the role of myocardin as a regulator of the smooth muscle differentiation pathway, but our results indicate that myocardin also plays a central role in the pathway leading to myocardial development. During *Xenopus* development, expression of myocardin is initially

detected in the cardiac primordia at stage 24, immediately prior to the onset of expression of the earliest myocardial differentiation markers (stage 25). The domain of myocardin expression coincides precisely with that of the differentiation markers (Fig. 2A). In contrast, several other important myocardial regulatory factors, including Nkx2-5, Gata4 and Tbx5 are expressed in precardiac tissues from approximately the time of gastrulation onwards (Tonissen et al., 1994; Jiang and Evans, 1996; Horb and Thomsen, 1999). Since no expression of myocardial markers is observed until about 24 hours after gastrulation, it is clear that co-expression of these factors alone is not sufficient to activate the cardiomyogenic pathway in vivo. Based on the expression profile therefore, it is highly likely that myocardin serves as an essential intermediate in the pathway leading to myocardial differentiation in the embryo.

Injection experiments show that expression of myocardin is able to activate high levels of expression of myocardial marker genes at ectopic locations in the *Xenopus* embryo (Fig. 3) or in animal cap tissue (Fig. 4). Ectopic expression of marker transcripts appeared to be most robust in neural tissues, but was observed at a variety of locations in the embryo, with the exception of endodermal tissue. Directed expression of myocardin in neural tissues using the *neural β-tubulin* promoter activated high levels of *MHCα* expression throughout neural tissues as late as the swimming tadpole stage. This indicates that myocardin is able to activate expression of at least some myocardial markers in the absence of other cardiac-specific transcription factors and without subverting pre-existing regulatory pathways. In the animal cap assays, myocardin initiated transcription of a number of smooth muscle markers, including *SM22*, *SM actin* and *CalpH1*. This is in agreement with previous studies that have demonstrated the ability of myocardin to activate endogenous smooth muscle genes in 10T1/2 fibroblasts and mouse embryonic stem cells (Du et al., 2003). Myocardin also activated a range of different myocardial marker genes in animal cap explants (Fig. 4). For all of these differentiation markers, significant levels of transcript had already accumulated by the gastrulation stage (stage 12.5) much earlier than the earliest differentiation in the intact embryo (stage 25). This observation suggests that myocardin can over-ride the normal temporal program of cardiac or smooth muscle development and cause the immediate activation of target genes. It is important to note however, that we never observed striated structures or beating tissue at ectopic locations in the myocardin-injected embryos, or in animal cap explants, indicating that myocardin alone is not sufficient to activate the complete pathway leading to myocardial differentiation in this context.

The observed ability of myocardin to cause ectopic transcription of myocardial markers is not a common property of cardiac transcription regulators. For example, numerous experiments have attempted to activate marker expression in the whole embryo or in animal cap explants, with Nkx2-5 and GATA factors, either alone or in combination (Cleaver et al., 1996; Chen and Fishman, 1996; Fu and Izumo, 1995; Jiang and Evans, 1996). In all cases, marker gene expression was either absent or extremely weak. Similarly, none of these transcription factors were capable of activating detectable expression of *MHCα* in our experiments. The recent observation that Nkx2-5 is an upstream regulator of myocardin (Ueyama et al., 2003) suggests that instances where Nkx2-5 overexpression successfully triggered cardiac marker expression (Chen and Fishman, 1996; Fu and Izumo, 1995) may have occurred via myocardin activation. An important exception to the preceding discussion is the recent observation that Gata4 is capable of generating beating cardiac tissue in animal cap explant cultures (Latinkic et al., 2003). In this case however, induction of the cardiogenic program requires nearly 10-fold higher levels of Gata4 mRNA than the amounts of myocardin mRNA used in our experiments (Figs 4, 5) and differentiation marker expression only occurs after extended culture. A plausible explanation for these results is that Gata4 initiates a cascade of events resulting in cardiac differentiation, while myocardin directly switches on transcription from target promoters.

Myocardin cooperates with other cardiac regulatory factors

Although several cardiac marker genes were transcriptionally activated in response to myocardin expression in embryos and animal caps, we were never able to detect expression of the *MLC2* gene. This is surprising since myocardin is able to activate transcription from the MLC2 promoter in COS cells (Wang et al., 2001; Wang et al., 2002). Previous studies have shown that transcription of myocardial genes is often regulated by cooperative interactions between transcription factors. For example, interactions between SRF, Nkx2-5, Gata4 and Tbx5 are known to be important for maximal expression from the *ANF* and *cardiac α-actin* promoters (Chen and Schwartz, 1996; Durocher and Nemer, 1998; Lee et al., 1998; Bruneau et al., 2001). Our experiments show that interactions with other transcription factors may also be important for myocardin activity, since co-expression of Gata4 with myocardin results in the induction of *MLC2* expression (Fig. 5). Although this observation is consistent with direct interactions of the myocardin and Gata4 proteins, we cannot exclude the possibility that Gata4 activates expression of other transcription factor(s), which then cooperate with myocardin to regulate MLC2. Previous studies have suggested that dimerization of myocardin is required for transcriptional activity, and that dimerization is facilitated by the presence of multiple SRF binding sites (CArG boxes), in the target promoter (Wang et al., 2003). We note that the *Xenopus* MLC2 gene contains two CArG boxes in the promoter region (Latinkic et al., 2004), but that, in this instance, myocardin requires cooperation with Gata4 to activate transcription from the *MLC2* promoter.

Myocardin loss-of-function and the genetic pathway to heart development

Previous studies using dominant negative versions of myocardin in *Xenopus* embryos resulted in the elimination of heart differentiation (Wang et al., 2001) suggesting an essential role for myocardin in cardiac development. However, mouse embryos lacking myocardin activity develop a fairly normal heart and die of vascular defects, presumably resulting from loss of vascular smooth muscle differentiation (Li et al., 2003). The relatively mild cardiac phenotype in the myocardin knockout mouse could be due to redundancy with the myocardin related factors (MRTF-A and MRTF-B), which are expressed in the developing heart in mice and possess similar

transcriptional properties (Wang et al., 2002). Since the MRTF-A and MRTF-B orthologues are not expressed in the developing *Xenopus* heart (Fig. 2G-I) we were able to use antisense morpholino knockdown methods to determine the role of myocardin in heart development, in the absence of rescuing activities. In these experiments, expression of the myocardial markers, *MHCα* and *MLC2* was dramatically reduced or eliminated using two different morpholinos (Fig. 6C and Table 1). This result is consistent with previous experiments in which expression of a dominant negative form of myocardin eliminated cardiac differentiation in the *Xenopus* embryo (Wang et al., 2001). Furthermore, myocardin MO-treated embryos show disruption of the normal morphological movements associated with heart tube formation (Fig. 6D). Overall, these results indicate an essential role for myocardin in *Xenopus* heart development and suggest that cardiogenesis in myocardin-null mice is partially rescued by redundant activities of MRTF-A and/or MRTF-B.

One of the unresolved questions relating to myocardin activity is the mechanism by which tissue-specific expression of target genes is regulated. In relatively naïve cells like *Xenopus* animal cap cells (Fig. 4) or mouse ES cells (Du et al., 2003), myocardin activates transcription of both cardiac and smooth muscle genes. Depending on the particular cell line in which myocardin is expressed, a different profile of smooth muscle and cardiac differentiation markers may be activated (Chen et al., 2002; Du et al., 2003; Wang et al., 2003). During normal embryonic development however, activation of myocardin target genes appears to be almost completely tissue specific. For example, expression of the smooth muscle marker, SM22, is never observed in the heart of *Xenopus* embryos (data not shown), but SM22 expression is highly activated by myocardin in *Xenopus* animal cap cells, together with a number of myocardial markers. Other authors have previously proposed that myocardin interacts with additional, tissue-specific transcription factors to modulate, either positively or negatively, its transcriptional activity (Du et al., 2003; Ueyama et al., 2003; Wang et al., 2003). It is interesting to observe therefore, that co-expression of myocardin with three additional cardiogenic transcription factors, Gata4, Nkx2-5 and Tbx5, in animal cap explants activated expression of both cardiac and smooth muscle markers (Fig. 5A). This result implies that none of these transcription factors is sufficient for suppression of smooth muscle gene expression in the heart. Identification of the proteins that help to specify myocardin target selection will be a key step towards understanding the cardiac and smooth muscle regulatory pathways.

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